The effect of iron-chelating agents on Magnetospirillum magneticum strain AMB-1: stimulated growth and magnetosome production and improved magnetosome heating properties

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The effect of iron chelating agents on *Magnetospirillum magneticum* strain AMB-1: stimulation of growth, magnetosome production and improvement of magnetosome heating properties.

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ABSTRACT

The introduction of various iron chelating agents to the AMB-1 bacterial growth medium stimulates the growth of AMB-1 magnetotactic bacteria and enhances the production of magnetosomes. After seven days of growth, the number of bacteria and the production of magnetosomes are increased in the presence of iron chelating agents by factors of up to ~ 2 and ~ 6, respectively. The presence of iron chelating agents also produces an increase of the magnetosome sizes and magnetosome chain lengths and yields an improvement of the magnetosome heating properties. The specific absorption rate (SAR) of suspensions of chains of magnetosomes isolated from AMB-1 magnetotactic bacteria, measured under the application of an alternating magnetic field of average field strength ~ 20 mT and frequency 198 kHz, increases from ~ 222 W/g_{Fe} in the absence of iron chelating agent up to ~ 444 W/g_{Fe} in the presence of 4 μM rhodamine B and ~ 723 W/g_{Fe} in the presence of 4 μM EDTA. These behaviors are observed for an iron concentration of 20 μM and iron chelating agent concentration lying below 40 μM.

INTRODUCTION

For a number of applications, it is advantageous to use large, single magnetic domain and well-crystallized magnetic nanoparticles, which possess a ferrimagnetic behavior at room temperature and are not prone to aggregation (Alphandéry et al 2011). Indeed, these types of nanoparticles possess a magnetic moment, which is stronger and more stable, than that of the smaller superparamagnetic magnetic nanoparticles, usually tested in biotechnological applications. Whereas monodomain nanoparticles, which are ferrimagnetic at room temperature, can hardly be produced chemically (Borderon et al 2011), certain species of bacteria, called magnetotactic bacteria (MTB), synthesize them. The organelles made of vesicles embedding magnetic crystals are called magnetosomes. They are usually composed of magnetite in the bacteria but they can oxidize into maghemite after their extraction from the bacteria due to their contact with oxygen (Alphandéry et al 2008). In addition, the magnetosomes isolated from the bacteria are not prone to aggregation due to their chain arrangement and the lipidic bi-layered membrane surrounding the magnetic crystal (Alphandéry et al 2011, Komeili 2006). MTB use the magnetosomes as a compass to navigate in the direction of the Earth magnetic field and to presumably find with increased efficiency the optimum environment for their growth and survival (Bazylinski et al 2004). The magnetosomes have already been shown to be useful for a number of applications in the commercial, scientific or medical fields. For example, they can be used to extract DNA, to magnetically detect biomolecular interactions or to separate cells (Arakaki et al 2005). The anti-tumoral activity of a complex formed by bacterial magnetosomes and doxorubicin has also been demonstrated experimentally (Sun et al 2007). Chains of magnetosomes extracted from AMB-1 magnetotactic bacteria have also been shown to be efficient to eradicate tumors. For that, they have been administered within tumors xeno-grafted under the skin of mice and heated under the application of an alternating magnetic field. In several mice, this treatment produced the disappearance of the tumor one month following the treatment (Alphandéry et al 2011). To consider a commercial application of the magnetosomes, the production yield of the MTB still needs to be increased. It has recently been improved in several species of magnetotactic bacteria including *Magnetospirillum magneticum* strain

AMB-1 (Matsunaga et al 1990, Matsunaga et al 1996a, Matsunaga et al 1996b, Matsunaga et al 2000, Yang et al 2001a, Yang et al 2001b, Yang et al 2001b), *Magnetospirillum magnetotacticum* strain MS-1 (Kundu et al 2010) and *Magnetospirillum gryphiswaldense* strain MSR-1 (Guo et al 2011, Heyen et al 2003, Lang et al 2006, Liu et al, 2010, Sun et al, 2008). To date, the highest production yield, which has been achieved, is 55 mg of magnetosomes synthesized per liter of growth medium and per day (Liu et al, 2010).

In this article, we present a method, which can be used to stimulate the growth of MTB and to improve their production yield. This method uses iron chelating agents, which are introduced to the bacterial growth medium of AMB-1 magnetotactic bacteria. A series of different iron chelating agents are tested. In addition, we also show that the introduction of iron chelating agents to the bacterial growth medium yields magnetosomes with increased magnetosome sizes and magnetosome chain lengths and results in improved magnetosome heating properties under the application of an alternating magnetic field.

MATERIALS AND METHODS

MTB belonging to the species *Magnetospirillum magneticum*, strain AMB-1, available at the ATCC under the reference 700264, have been cultivated in a volume of either 10 ml or 500 ml. A suspension containing either 100 μl of ~ 5.10^8 bacteria or 5 ml of ~ 2.10^9 bacteria was first inoculated in 10 ml or 500 ml of ATCC bacterial growth medium 1653 respectively. The cells were cultivated in micro-anaerobic conditions, *i. e.* in a culture medium that had not been degased, but that was closed and thus not in contact with air. The culture of MTB was carried out in an incubator at 26 °C using a slightly modified ATCC medium 1653, whose composition is given by the ATCC. The pH of the culture medium was adjusted to 6.85 by using a 5 M solution of sodium hydroxide. The suspensions containing the various chelating agents (EDTA, ethylenediaminetetracetic acid, rhodamine B, ascorbic acid, erythrosine, anthranilic acid, citric acid, 3-(N-morpholino)propanesulfonic acid, 3-(cyclohexylamino)-1-propanesulfonic acid, calcein, dextran, alendronic acid, neridronic acid, nicotinamide) mixed in water

90   were then inserted within the bacterial growth medium. The concentration of iron chelating agents

91   contained within the bacterial growth medium was varied between 0.4 μM and 400 μM.

92   After incubation, two different types of suspensions containing either whole inactive MTB or extracted

93   chains of magnetosomes mixed in water were prepared. To prepare the suspensions containing the

94   whole MTB, the cells were harvested by centrifugation at 4 000 g during 20 minutes at room

95   temperature. The supernate was removed and the cells were resuspended in milli-Q water. To extract the

96   chains of magnetosomes, 2 ml of cell suspension obtained as previously described was centrifugated

97   again and resuspended in a 10 ml tris-HCl buffer of pH 7.4. The cellular suspension was then sonicated

98   during 120 minutes at 30 W in order to lyse the cells and collect the chains of magnetosomes. After

99   sonication, the suspension containing the chains of magnetosomes was separated from the cellular debris

100   by positioning a strong neodymium magnet (0.1-1 T) next to the tube, and the magnetic material was

101   then harvested. The supernate containing the cellular debris and other organic molecules was eliminated.

102   The chains of magnetosomes were washed 10 to 20 times in deionized water at pH 7.4 and were then

103   resuspended in 500 μL of milli-Q water.

104   In order to obtain a sufficiently large amount of chains of magnetosomes, the MTB were first cultivated

105   in a volume of 500 ml either in the absence or in the presence of various chelating agents. They were

106   centrifugated (4000 g, 20 min) after 7 days of growth. The supernate was then removed and the bacteria

107   were resuspended in 2 ml of mili-Q-water. For the various suspensions of MTB, the absorption was

108   measured at 565 nm (Heyen et al 2003) and the number of bacteria was estimated using a Beckman

109   Coulter Z1 DT. The concentrations in maghemite of the various suspensions of extracted chains of

110   magnetosomes were measured by absorption at 480 nm (Alphandéry et al 2011).

111   The growth curves of the MTB grown either in the absence or in the presence of the various iron

112   chelating agents were measured for the MTB cultivated in 10 ml elongated tubes allowing the presence

113   of an oxygen gradient similar to that encountered in natural environments. The bacteria were

114   centrifugated 1 day, 2 days, 3 days, 4 days, 7 days, 8 days and 9 days following the inoculation of the

5

115   MTB. The supernate was removed and the bacteria were resuspended in 1 ml of mili-Q-water. The

116   growth curves of the various suspensions of whole MTB were then measured during the ten days

117   following the inoculation by measuring both the absorption at 565 nm (Heyen 2003) and the number of

118   bacteria using a Beckman Coulter Z1 DT. In order to evaluate the presence (or not) of the

119   magnetosomes within the bacterial growth medium, the magnetic moments of the different suspensions

120   of magnetotactic bacteria were measured. For that, 600 µl of the various suspensions of MTB were

121   deposited on top of non magnetic absorbing paper, which was then inserted within a capsule of gelatin.

122   This capsule was positioned in a SQUID. The magnetic moment of the MTB was estimated under the

123   application of a magnetic field of 1000 Oe.

124   Transmission electron microscopy (TEM) studies were carried out using a JEOL 2100F with field

125   emission gun. For the TEM measurements, 5 µl of a suspension of whole MTB was deposited on top of

126   a copper grid covered with a thin amorphous carbon film. TEM measurements were used to measure the

127   sizes of the magnetosomes and to estimate the lengths of the chains of magnetosomes in bacteria

128   cultivated either in the absence or in the presence of iron chelating agents.

129   The maghemite composition of the magnetosomes was determined by measuring the saturation

130   isothermal remanent magnetization curves of the magnetotactic bacteria cultivated in various conditions

131   following the same method as that described previously (Alphandéry et al 2008). The absence of the

132   Verwey transition indicates that the magnetosomes are oxidized into maghemite.

133   The heating curves of suspensions containing 300 µl of extracted chains of magnetosomes mixed in

134   water with a concentration in maghemite of 478 µg/ml were measured. The maghemite composition of

135   the chains of magnetosomes extracted from AMB-1 magnetotactic bacteria was estimated following a

136   method previously described (Alphandéry et al 2008). The concentration in maghemite of the different

137   suspensions of extracted chains of magnetosomes was measured at 480 nm. To generate heat, the

138   suspensions of extracted chains of magnetosomes were exposed to an alternating magnetic field of

6

139   frequency 198 kHz and average field amplitude of 20 mT using a 10 kW EasyHeat power supply from

140   Ambrell, Soultz, France. The average field strength within the coil was measured using a 2 D probe

141   (Suppl. Fig. 1). The temperature of the suspensions was measured using a thermocouple microprobe (IT-

142   18, Physitemp, Clifton, USA).

143   **RESULTS**

144   Figure 1 shows the optical absorption, measured at 565 nm, of suspensions of MTB cultivated either in

145   the absence or in the presence of various iron chelating agents and harvested after 7 days of growth.

146   About 70 % of the iron chelating agents tested produces an increase of the absorption of the suspension

147   of MTB. This indicates that in most cases the presence of iron chelating agents within the bacterial

148   growth medium stimulates the growth of the MTB. The growth stimulation is observed at low

149   concentrations of iron chelating agents, which lie below 40 μM (Figure 1). The most pronounced growth

150   stimulations are observed for hemoglobin at concentration 40 μM, citric acid at concentration 4 μM, and

151   hemoglobin at concentration 4 μM for which the optical absorptions of the suspension of MTB is 1.5 to

152   2.5 times larger than that of the bacteria cultivated in the absence of iron chelating agents. Figure 2

153   shows the concentration in maghemite of the suspensions containing chains of magnetosomes extracted

154   from whole MTB harvested after seven days of growth and cultivated in the same conditions as those

155   presented in Figure 1. Since the magnetosomes studied here are oxidized into maghemite (Alphandéry

156   2008), the concentration in maghemite, which is measured, is equal to the concentration in

157   magnetosomes. We therefore deduce from Figure 2 that the production of magnetosomes is increased

158   for a majority of iron chelating agents tested (~ 65 %) and for concentrations in iron chelating agents,

159   which lie below 40 μM. The strongest enhancement of the magnetosome production is observed when

160   0.4 μM or 4 μM of hemoglobin is introduced within the bacterial growth medium. In this case,

161   enhancements of the magnetosome production by factors of 6 are achieved. It is also worth mentioning

162   that the concentration in iron chelating agent, which produces the largest growth stimulation, for

163 example 40 µM for hemoglobin (Figure 1), is not always the same as that, which yields the largest

164 magnetosome production (for example 4 µM for hemoglobin).

165 The influence of introducing iron chelating agents to the bacteria growth medium on several MTB and

166 magnetosome properties is studied next. We describe the results obtained with an iron chelating agent,

167 rhodamine B, which shows a typical behavior, *i. e.* which stimulates well both the growth of the MTB

168 and the production of the magnetosomes. Figure 3(a) shows the growth curves of MTB cultivated either

169 in the absence of rhodamine B or in the presence of different concentrations of rhodamine B. In the

170 presence of 0.4 µM, 4 µM or 40 µM of rhodamine B introduced to the bacterial growth medium, the

171 growth of MTB is more rapid than in the absence of rhodamine B. The concentration of 400 µM of

172 rhodamine B does not stimulate the growth of MTB. The growth stimulation induced by the presence of

173 rhodamine B is significant during the 3 or 4 first days of growth, *i. e.* during the exponential phase. After

174 that, the bacteria reach the stationary phase and then the absorption of the different suspensions of

175 bacteria decreases most probably due to the death of the bacteria (Suppl. Fig. 2). In order to determine

176 the rate at which the magnetosomes grow, the magnetic moments of the suspensions of MTB cultivated

177 in the absence or in the presence of various concentrations of rhodamine B were measured for an applied

178 magnetic field of 1000 Oe. Before the fourth day of growth, the magnetic moments of the various

179 suspensions of bacteria were not detected indicating the absence or undetectable concentrations of

180 magnetosomes in these suspensions. At days 4, 7 and 8, the magnetic moments of the suspensions

181 containing MTB cultivated in the presence of 0.4 µM, 4 µM or 40 µM of rhodamine B were detected.

182 The magnetic moments of the suspensions containing MTB cultivated in the absence of rhodamine B

183 were detected at a later stage during the ninth day of growth, indicating that the production of the

184 magnetosomes is more rapid for the MTB cultivated in the presence of rhodamine B than for those

185 cultivated in the absence of rhodamine B. The stimulations of the growth of the bacteria and of the

186 production of the magnetosomes were observed for other iron chelating agents introduced within the

187 bacterial growth medium, such as ascorbic acid (Suppl. Fig. 3).

188 The magnetosome sizes and magnetosome chain lengths of the extracted chains of magnetosomes were

189 measured for the MTB cultivated in the absence of rhodamine B and for those cultivated in the presence

190 of 4 μM of rhodamine B (Figure 4). The distributions in magnetosome sizes and magnetosome chain

191 lengths are bimodal. For the magnetosomes of small sizes, the magnetosome sizes and magnetosome

192 chain lengths increase from an average of ~ 27.5 nm and ~ 150 nm in the absence of iron chelating agent

193 up to an average of ~ 35 nm and ~ 200 nm in the presence of 4 μM rhodamine B, respectively (Figures

194 4(a)-(d)). The largest magnetosomes of the longest chains increase from an average of ~ 42.5 nm and ~

195 750 nm in the absence of rhodamine B up to an average of ~ 65 nm and ~ 800 in the presence of

196 rhodamine B, respectively (Figures 4(a)-(d)). The increase of the magnetosome sizes and magnetosome

197 chain lengths was also observed in the presence of other iron chelating agents such as EDTA (Suppl.

198 Fig. 4).

199 Three suspensions containing 300 μl of extracted chains of magnetosomes with a concentration in

200 maghemite of 478 μg/ml were then heated under the application of an alternating magnetic field of

201 frequency 198 kHz and average magnetic field strength of 20 mT. These suspensions contain chains of

202 magnetosomes synthesized either in the absence or in the presence of 4 μM Rhodamine B or 4 μM

203 EDTA. Figure 5(a) shows that the absorption curves of the three suspensions are the same, indicating

204 that their concentration is identical. The variations of temperature as a function of time of the three

205 suspensions are shown in Figure 5(b). The increase in temperature is more important in the presence of

206 4 μM rhodamine B or 4 μM EDTA than in the absence of iron chelating agent. The slopes, measured at

207 25 °C, of the variations of temperature with time shown in Figure 5(b), designated as $\Delta T/\delta t$, increase

208 from $\Delta T/\delta t \sim 0.005$ °C/sec. in the absence of iron chelating agent up to $\Delta T/\delta t \sim 0.011$ °C/sec in the

209 presence of 4 μM Rhodamine and 0.017 °C/sec in the presence of 4 μM EDTA. Using the relation

210 between the specific absorption rate (SAR) and ($\Delta T/\delta t$), which is given by SAR = $C_v(\Delta T/\delta t)$, where C_v =

211 4.2 J/(g.K) is the specific heat capacity of water, we find that the SAR increases from ~ 222 W/g_{Fe} in the

212   absence of iron chelating agent up to ~ 444 W/g_{Fe} in the presence of 4 μM rhodamine B and ~ 723

213   W/gFe in the presence of 4 μM EDTA.

214   **DISCUSSION**

215   We have shown that the presence of iron chelating agents within the bacterial growth medium stimulates

216   the production of the magnetosomes. The function of the iron chelating agents is to chelate Fe^{3+} iron

217   ions and to enhance the bioavailability of iron by forming Fe^{3+}-ligand complexes. The iron chelating

218   agents could be involved in one or several of the following steps of the magnetosome formation, (i) the

219   formation of the vesicle in which the magnetosomes are formed, (ii) the iron uptake by the cells, (iii) the

220   iron transport within the cells and (iv) the control of the biomineralization of the magnetosome

221   formation in Fe_3O_4 or γFe_2O_3 (Bazylinski 2004). They could therefore favor the magnetosome formation

222   by making each or several of these steps more efficient.

223   We examine why the presence of iron chelating agents stimulates the growth of the bacteria. The

224   mechanisms, which enhance the production of the magnetosomes and those, which stimulate the growth

225   of the bacteria, are expected to be different. The MTB are believed to synthesize siderophores, which

226   help to incorporate iron within the bacteria (Calugay 2006). In the absence of such complexing agents,

227   Fe^{3+} iron ions precipitate mostly as ferrihydrite and their bioavailability is likely strongly decreased.

228   When iron chelating agents are introduced to the bacterial growth medium, the MTB have less need to

229   synthesize siderophores. It is thus likely that the energy allocated for siderophore synthesis can then be

230   used for bacterial growth. This could explain the more efficient growth observed in the presence than in

231   the absence of iron chelating agents.

232   We also observe that the highest stimulation of the bacteria is observed for large iron chelating agents,

233   such as hemoglobin. This result suggests that iron is chelated in a more efficient way using high

234   molecular weight than low molecular weight iron chelating agents. This is in agreement with previous

235   study (Reichard *et al*, 2007). There are several possible explanations for this behavior. The stability

236 formed by a large iron complex could be higher than that formed by a smaller iron complex. A large iron

237 complex may also more easily enter within the periplasm of the cells. It is also possible that a large iron

238 complex would more easily release iron within the bacterial cells or that it would favor oxidoreduction

239 reactions involved in the magnetosome production or crystallization process.

240 We have shown that above a concentration in iron chelating agent lying between 40 µM and 400 µM,

241 the stimulation of the growth of the MTB and of the production of the magnetosomes is not observed.

242 This result agrees with previous reports, which showed that for a ratio of 100 to 500 between the iron

243 chelating agent and iron concentrations, no growth stimulation was observed for the species MSR-1

244 (Schüler 1996). This indicates that the iron chelating agents are toxic above concentrations lying

245 between 40 µM and 400 µM. We propose two hypotheses to explain this result. Either a high iron

246 chelating agent concentration yields a high iron concentration within the bacterial growth medium,

247 which is toxic for the magnetotactic bacteria. Or the reactive oxygen species, which are formed because

248 of the alcohol functional groups getting unbound from iron, for example in rhodamine B, damage the

249 membrane of the bacteria.

250 The kinetics of the magnetosome formation is reported for the AMB-1 species. For this species, the

251 magnetosomes are probably mainly formed after the magnetotactic bacteria have reached the stationary

252 phase (Yang 2001). We have shown that the introduction of iron chelating agents within the bacterial

253 growth medium of AMB-1 magnetotactic bacteria yields faster growth of magnetosomes. This should

254 therefore enable a significant increase in the rate of production of the magnetosomes by the AMB-1

255 magnetotactic bacteria. In strain MSR-1, the magnetosomes are produced earlier, before the bacteria

256 have reached the stationary phase (Heyen and Schüler 2003). This behavior could be explained by a

257 different mechanism of iron uptake or of magnetosome formation between these two species of

258 magnetotactic bacteria. Therefore, the influence of introducing iron chelating agents within the bacterial

259 growth medium on growth rate of magnetosome production may be less pronounced for the MSR-1 than

260 for the AMB-1 species.

261 The introduction of iron chelating agents to the bacterial growth medium also increases the

262 magnetosome sizes and magnetosome chain lengths, which results in an improvement of the

263 magnetosome heating properties. The specific absorption rate of suspensions of extracted chains of

264 magnetosomes increases by a factor of 3 between the MTB cultivated in the absence of iron chelating

265 agent and those cultivated in the presence of 4 μM EDTA. High values of SAR are advantageous to

266 carry out magnetic hyperthermia treatments of cancers since they enable the use of a small quantity of

267 magnetosomes and/or the application of an alternating magnetic field of low strength, hence decreasing

268 the risks of toxicity.

269 Further studies should explore separately the effect of iron on either the bacterial growth or the

270 production of magnetosomes.

271 In conclusion, the introduction of iron chelating agents within the bacterial growth medium enables the

272 stimulation of the growth of the MTB, the enhancement of the production yield of the magnetosomes,

273 the increase of the magnetosome sizes and magnetosome chain lengths as well as the enhancement of

274 the magnetosome heating properties under the application of an alternating magnetic field.

275 **ACKNOWLEDGMENTS**

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FIGURES

348 Figure 1: Absorption measured at 565 nm and number of magnetotactic bacteria per ml of suspensions

349 of magnetotactic bacteria cultivated either in the absence of iron chelating agents or in the presence of

350 0.4 μM, 4 μM, 40 μM or 400 μM of various iron chelating agents. These chelating agents are EDTA,

351 rhodamine B, ascorbic acid, erythrosine, anthranilic acid, citric acid, 3-(N-morpholino)propanesulfonic

352 acid, 3-(cyclohexylamino)-1-propanesulfonic acid, calcein, dextran, alendronic acic, neridronic acid and

353 nicotinamide. Dotted lines represent control values.

354 Figure 2: The concentration in maghemite of suspensions containing chains of magnetosomes extracted

355 from whole magnetotactic bacteria. The magnetotactic bacteria have been cultivated either in the

356 absence or in the presence of the same iron chelating agents as in Figure 1.

357 Figure 3: (a) The growth curves of magnetotactic bacteria cultivated either in the absence of rhodamine

358 B or in the presence of 0.4 μM, 4 μM, 40 μM or 400 μM of rhodamine B. The growth curves represent

359 the absorption of the different suspensions of magnetotactic bacteria at 565 nm (left y scale) or the

360 number of MTB per ml (right y scale) measured at the different days of growth (day 0, day 1, day 3, day

361 4). (b) The Magnetic moments of the suspensions of magnetotactic bacteria cultivated either in the

362 absence or in the presence of 0.4 μM, 4 μM, 40 μM or 400 μM of rhodamine B, measured under the

363 application of a magnetic field of 1000 Oe during the fourth day, the seventh day, the eighth day or the

364 ninth day of bacterial growth.

365 Figure 4: (a), (b) The magnetosome size distribution of the magnetosomes contained within the

366 magnetotactic bacteria for the bacteria cultivated either in the absence, (a), or in the presence of 4 μM

367 rhodamine B, (b). (c), (d) The magnetosome chain length distribution of the chains of magnetosomes

368 contained within the magnetotactic bacteria for the bacteria cultivated either in the absence, (c), or in the

369 presence of 4 μM rhodamine B, (d).

370 Figure 5: (a) The absorption curves of three suspensions of extracted chains of magnetosomes. The

371 MTB were cultivated either in the absence of iron chelating agent, in the presence of 4 μM rhodamine B

372 or in presence of 4 μM EDTA. (b) The variations of temperature as a function of time of the same

373 suspensions as in (a) exposed to an alternating magnetic field of frequency 198 kHz and average field

374 strength of 20 mT. Ch-Std, Ch-EDTA (4μM) and Ch-Rhodamine B (4μM) designate the chains of

375 magnetosomes isolated from the bacteria obtained by cultivating the bacteria in the absence of iron

376 chelating agent, in the presence of 4 μM EDTA or in the presence of 4 μM rhodamine B, respectively.

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Whole magnetotactic bacteria AMB-1 harvested after 7 days of growth
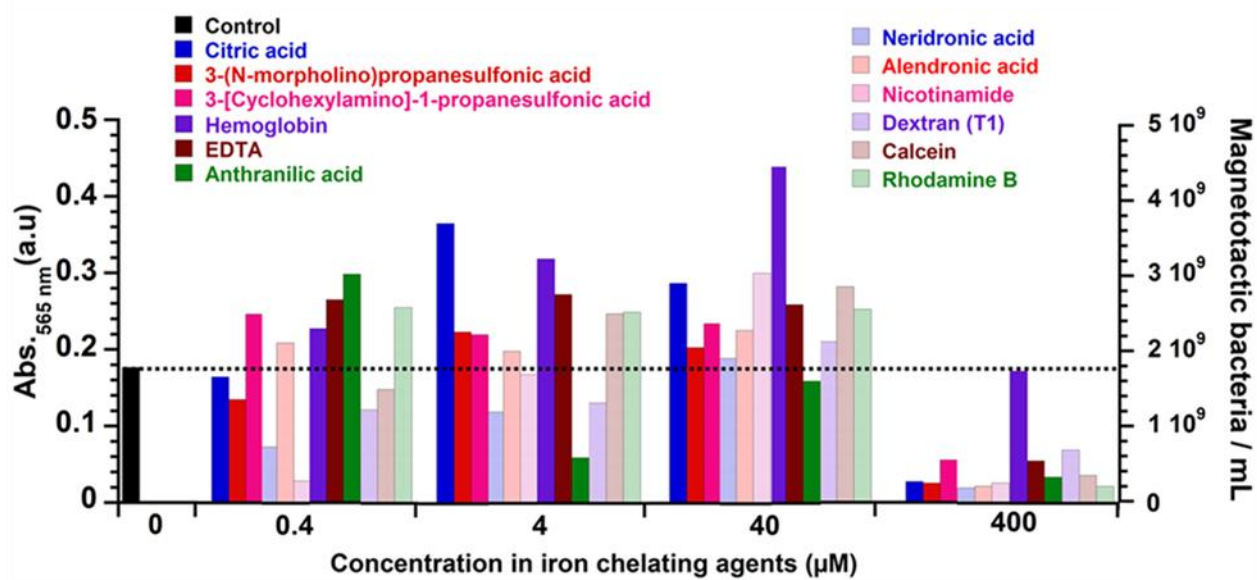
FIG. 1

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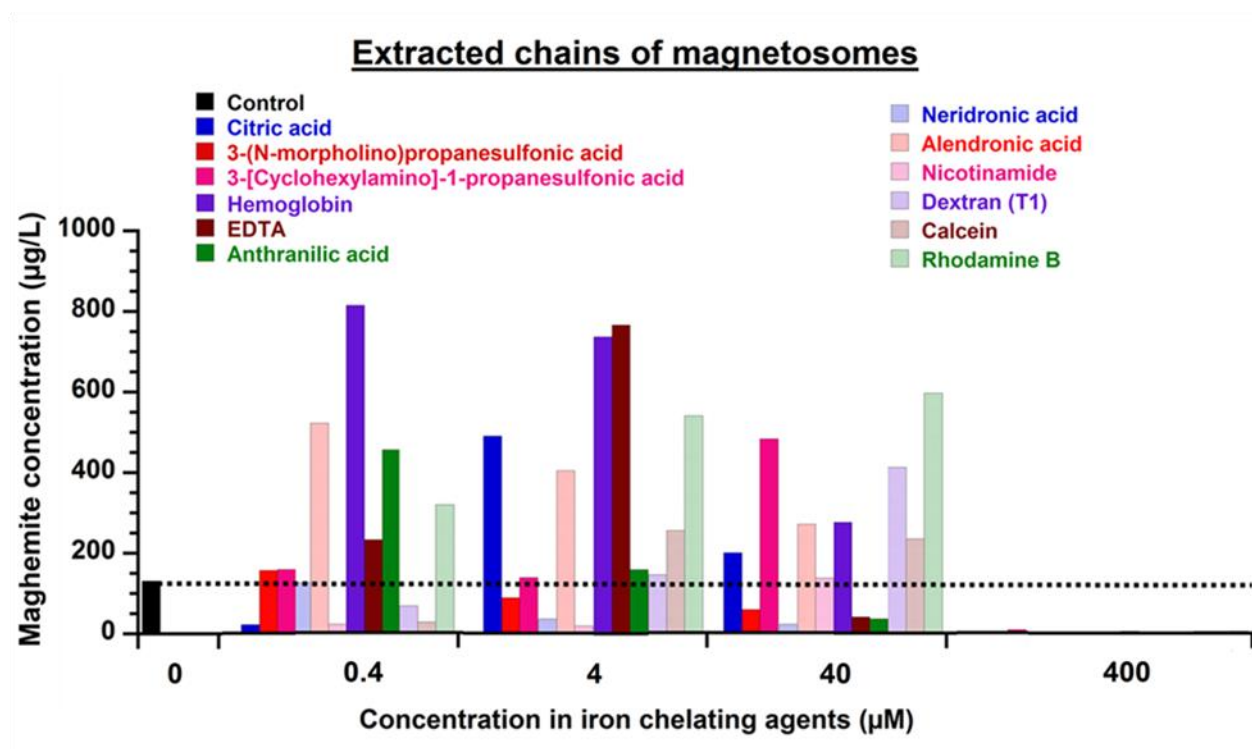388

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FIG. 2

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FIG. 3

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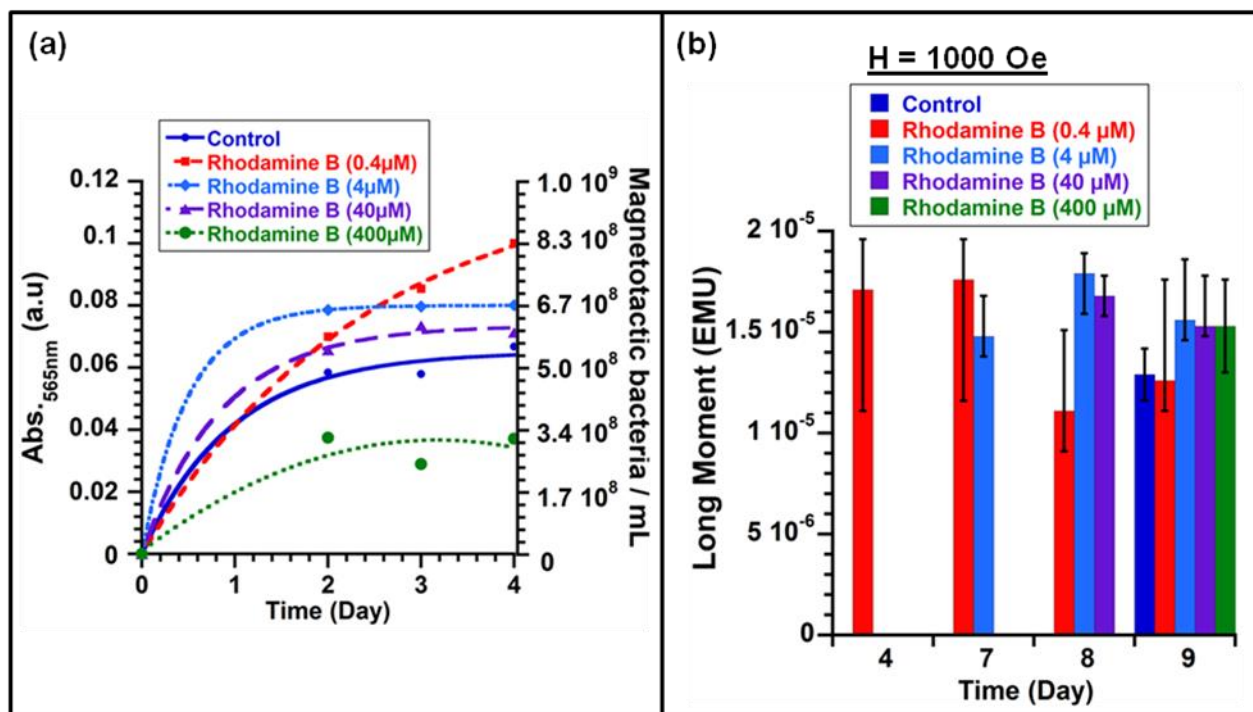
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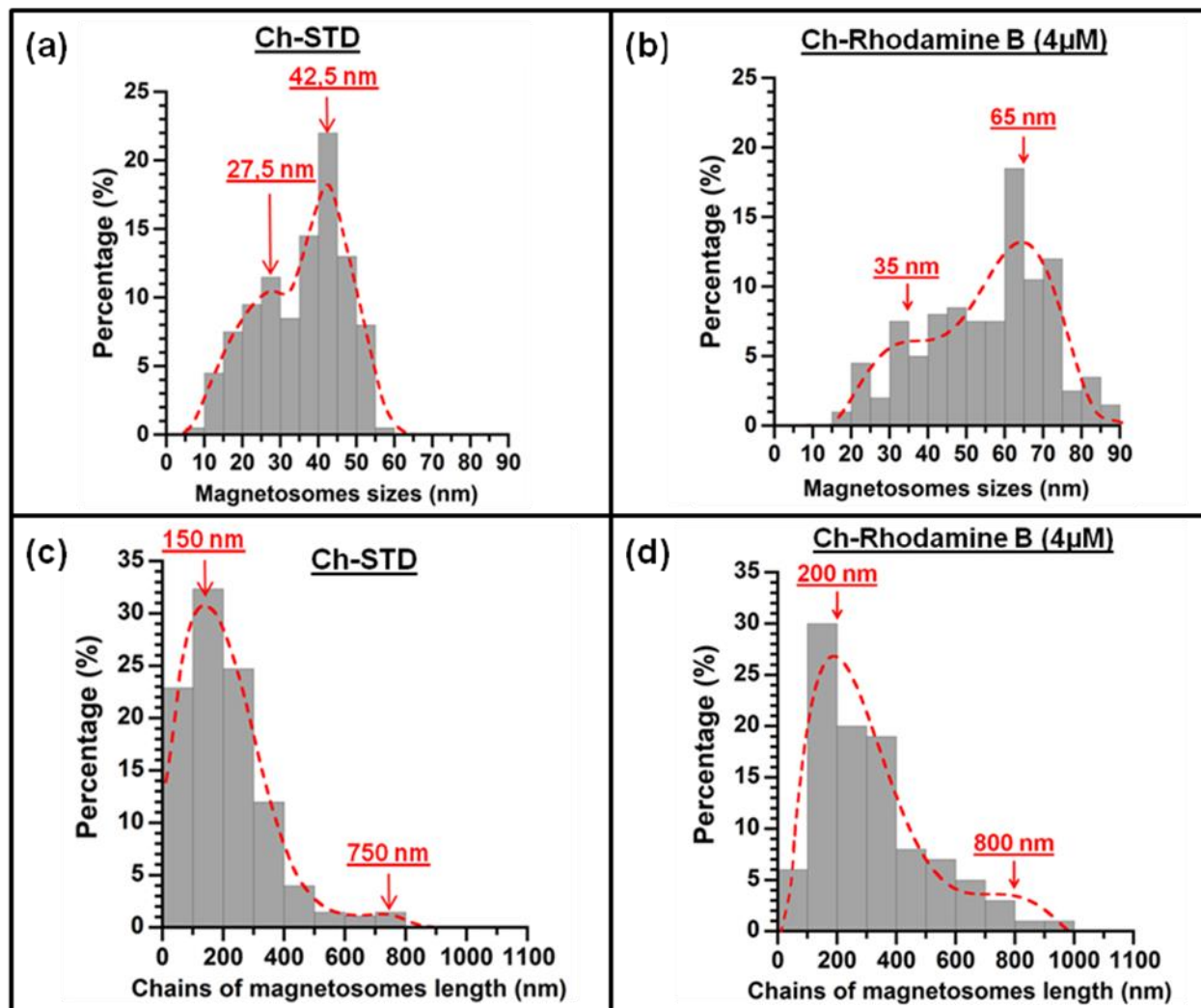
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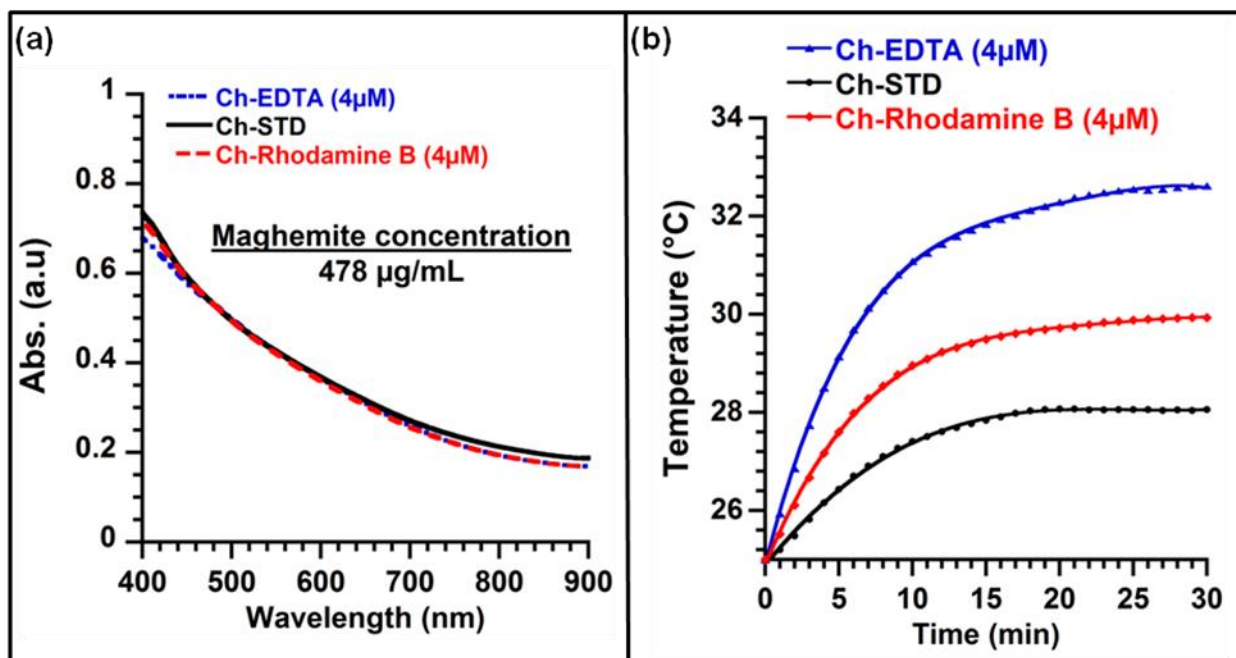
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FIG. 4

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FIG. 5

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